

Rolling Nagoya Mouse Strain (PROD-*rol/rol*) with Classic Piebald Mutation

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(Received 24 February 2014/Accepted 5 April 2014/Published online in J-STAGE 23 April 2014)

ABSTRACT. Ataxic *rolling* Nagoya (PROD-*rol/rol*) mice, which carry a mutation in the $\alpha 1$ subunit of the $\text{Ca}_v2.1$ channel (*Cacna1a*) gene, were discovered in 1969. They show white spots on agouti coat and have a mutation in the piebald spotting (*s*) locus. However, mutation analysis of the *s* locus encoding the endothelin receptor type B (*Ednrb*) gene in PROD-*rol/rol* mice had not been performed. Here, we examined the genomic and mRNA sequences of the *Ednrb* gene in PROD-*rol/rol* and wild-type *rolling* Nagoya (PROD-*s/s*) and studied the expression patterns of *Ednrb* and *Cacna1a* genes in these mice in comparison with C57BL/6J mice. Polymerase chain reaction analyses revealed two silent nucleotide substitutions in the coding region and insertion of a retroposon-like element in intron 1 of the *Ednrb* gene. Expression analyses demonstrated similar localizations and levels of *Ednrb* and *Cacna1a* expression in the colon between PROD-*rol/rol* and PROD-*s/s* mice, but the expression levels of both genes were diminished compared with C57BL/6J mice. Microsatellite genotyping showed that at least particular regions of chromosome 14 proximal to the *Ednrb* locus of the PROD strain were derived from Japanese fancy piebald mice. These results indicated that PROD-*rol/rol* mice have two mutant genes, *Ednrb* and *Cacna1a*. As no PROD strain had an intact *Ednrb* gene, using congenic *rolling* mice would better serve to examine *rolling* Nagoya-type $\text{Ca}_v2.1$ channel dysfunctions.

KEY WORDS: *Ednrb* gene, microsatellite genotyping, *M. m. molossinus*, piebald mutation, PROD-*rol/rol* mouse

doi: 10.1292/jvms.14-0096; *J. Vet. Med. Sci.* 76(8): 1093–1098, 2014

Autosomal recessive ataxic *rolling* Nagoya (PROD-*rol/rol*) mice, which exhibit white spots on the agouti coat, were found among descendants of a cross between the SIII and C57BL/6 strains and maintained by intercross mating [14]. The symbol for this mutant gene is “*rol*” based on the rolling symptoms with poor motor control that appear from 10 to 14 days after birth. The results of linkage analysis indicated that no relationship exists between “*rol*” and the mutant gene for the white spotting defect [14]. The “*rol*” gene mutation has been reported to be a point mutation in the $\alpha 1$ subunit of the voltage-gated Ca^{2+} (Ca_v) channel 2.1 (*Cacna1a*) gene on chromosome 8, causing an amino acid change at R1262G in the pore-forming $\text{Ca}_v2.1\alpha 1$ subunit ($\text{Ca}_v2.1\alpha 1$) channels and decreased $\text{Ca}_v2.1$ channel currents [12]. $\text{Ca}_v2.1\alpha 1$ is expressed mainly in the nervous system [10, 15] and controls neuronal functions, including neurotransmitter release [1, 2].

Piebald spotting (*s*) is a classical mouse mutation that affects melanocytes originating from the neural crest during development [7] and induces pigmented and white spots [11, 17]. The *s* locus on mouse chromosome 14 contains the endothelin receptor type B (*Ednrb*) gene [6]. According to the RIKEN BioResource Center Database (http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC00388), PROD-*rol/rol* mice show an irregular white-spotted coat, which is a similar phenotype to *piebald* mutant (*s/s*) mice

[5], and indeed, these mice have a mutation in the *s* locus. Studies have reported two silent nucleotide substitutions in the coding region and an insertion of a retroposon-like element in intron 1 of the *Ednrb* gene leading to reduced expression of *Ednrb* in the region of colon as the piebald (*s/s*) mutation [9, 20]. The same mutation in the *Ednrb* gene was detected in the Japanese fancy mouse 1 (JF1), which was reported to be derived from *Mus musculus molossinus* [9], and also shows irregular white spots on a black coat [8]. Moreover, at least a region adjacent to the *Ednrb* locus on chromosome 14 in JF1 and *s/s* mice has been suggested to be derived from *M. m. molossinus*. However, no detailed mutation analyses of the *Ednrb* gene have been performed in the PROD strain.

In this study, we examined the genomic and mRNA sequences of the *Ednrb* gene in PROD-*rol/rol* and wild-type *rolling* Nagoya (PROD-*s/s*) and also examined the expression patterns of *Ednrb* and *Cacna1a* genes in the colon of these mice compared with C57BL/6J mice. We also typed microsatellite loci adjacent to the *Ednrb* locus to examine the origin of the *Ednrb* allele of PROD mice.

MATERIALS AND METHODS

Animals: The research was conducted in accordance with the Declaration of Helsinki and was approved by the Animal Experiments Committee of RIKEN (Approved ID No. H24-2-206). The heterozygous *rolling* Nagoya (PROD-*rol/+*) strain was provided by the RIKEN BioResource Center (Tsukuba, Japan), with support from the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. C57BL/6J (B6) mice were purchased from Charles River Japan (Yokohama, Ja-

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pan). The homozygous *rolling* Nagoya (PROD-*rol/rol*) and wild-type *rolling* Nagoya (PROD-*s/s*) strains were bred by mating with PROD-*rol/+* mice [19]. The mice were allowed *ad libitum* access to water and food pellets (CRF-1; Oriental Yeast, Tokyo, Japan) and kept under conditions of controlled room temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) with a 12:12-hr light–dark cycle (lights on from 08:00 to 20:00). Separate groups of mice at age 3–5 weeks were used in this study.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNAs from the brains of PROD-*s/s* (male, female: $n=10, 10$), PROD-*rol/rol* (male, female: $n=10, 10$) and B6 (male, female: $n=10, 10$) mice were isolated using TRIzol reagent (Invitrogen, Burlington, Canada). First-strand complementary DNA (cDNA) was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen). The following PCR primer sets were used for amplification of *Ednrb* (GenBank ID: NM_007904.4): forward, 5'-GC-TAGTGTGTTTTCAGAGGCTTG-3', and reverse, 5'-GAC-TAAGAATCTAAGTGCTTCCC-3'. The RT-PCR products were sequenced using an ABI Prism 3730 (Applied Biosystems, Foster City, CA, U.S.A.). Alterations in the transcript structure were examined using the database sequence.

Genomic structure analysis: To distinguish normal alleles from those with insertion of a retroposon-like element in intron 1 of the *Ednrb* gene (GenBank ID: AB242436.1), the PCR products were amplified with genomic DNA from the tails PROD-*s/s* (male, female: $n=10, 10$), PROD-*rol/rol* (male, female: $n=10, 10$) and B6 (male, female: $n=10, 10$) mice, and allele-specific primers designed according to a previous report [4]. The PCR products were sequenced and examined for the presence or absence of the insertion.

Real-time quantitative RT-PCR (real-time qRT-PCR): The levels of *Ednrb* and *Cacna1a* mRNA were measured using Applied Biosystems TaqMan Gene Expression Assays (*Ednrb*, Assay ID: Mm01224433_m1; *Cacna1a*, Assay ID: Mm00432190_m1). All data were normalized relative to 18S ribosomal RNA (Assay ID: Hs99999901_s1, Applied Biosystems TaqMan Gene Expression Assay). The PCR conditions were 94°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. All male samples (PROD-*s/s*, PROD-*rol/rol* and B6: $n=\text{each } 10$) were analyzed in duplicate, and the threshold cycle (Ct) value was calculated to determine the relative levels of expression [19].

In situ hybridization: The male gastrointestinal tract (PROD-*s/s*, PROD-*rol/rol* and B6: $n=\text{each } 5$) was dissected after perfusion with saline followed by 4% paraformaldehyde, fixed with Tissue Fixative (Gonostaff, Co., Ltd., Tokyo, Japan), embedded in paraffin and cut into section at a thickness of $6 \mu\text{m}$ for *in situ* hybridization. The hybridization protocol was performed as reported previously [16]. The probes for the $\text{Ca}_v2.1 \alpha_1$ subunit cDNA fragment (positions 6068–6748) were labeled with a digoxigenin RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Color reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) overnight and then washed with phosphate-buffered saline (PBS). The sections were counterstained with Kernechtrot stain solution (Mutoh Pure

Chemicals, Tokyo, Japan) and mounted with CC/Mount (Diagnostic BioSystems Inc., Pleasanton, CA, U.S.A.).

Microsatellite genotyping: To examine the origin of the *Ednrb* allele from genomic DNA obtained from the tails of PROD-*s/s* (male, female: $n=10, 10$), PROD-*rol/rol* (male, female: $n=10, 10$) and B6 (male, female: $n=10, 10$) mice, microsatellite markers (*D14Mit30*, *D14Mit193*, *D14Mit93*, *D14Mit94*, *D14Mit170*, *D14Mit42* and *D14Mit267*) were typed by PCR amplification using the Mouse Microsatellite Data Base of Japan (MMDBJ, <http://www.shigen.nig.ac.jp/mouse/mmdbj/>) and the Mouse Genome Informatics (MGI, <http://www.informatics.jax.org/>).

Statistical analysis: The data are presented as the means \pm standard error of the mean (SEM). Statistical analyses were conducted using Excel Statistics 2006 (SSRI, Tokyo, Japan). The data were analyzed using Dunnett's test between groups where appropriate. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Gross observation of PROD strain: PROD-*s/s* and PROD-*rol/rol* mice had irregular white spots on the agouti coat (Fig. 1A). PROD-*rol/rol* mice showed poor motor coordination and stiffness of the hindlimbs. Neither PROD-*s/s* nor PROD-*rol/rol* mice showed the megacolon phenotype (Fig. 1B).

Sequence analysis of the *Ednrb* gene coding region: Two C to T nucleotide substitutions were found at nucleotides 159 and 1032 in the coding region of the *Ednrb* gene in PROD-*s/s* mice (Fig. 2A). However, the amino sequence of *Ednrb* in PROD-*s/s* mice was not different from the sequence of the B6 strain in the database. These silent nucleotide substitutions in the *Ednrb* gene were detected in *s/s* mice [6] and JF1 mice [9] as a piebald mutation. We found nucleotide substitutions in the *Ednrb* gene (Genbank ID: U32329.1) in both male and female PROD-*s/s* and PROD-*rol/rol* mice (data not shown).

Structure analysis of intron 1 of the *Ednrb* gene: Intron 1 has the 5.5-kb retroposon-like element in *s/s* mice [20] and JF1 mice [4]. The F and R-wt primers correspond to intron 1 of the *Ednrb* gene (Fig. 2A) and amplify a 225-base pair (bp) fragment as the wild type. The R-mu primer corresponds to a retroposon-like element in the *s/s* mutant (Fig. 2A), and the F and R-mu primers amplify a 318-bp fragment containing part of intron 1 of the *Ednrb* gene and the retroposon-like element of the *s/s* mutant type. Although a 225-bp fragment was amplified from B6 mice, PROD-*s/s* mice and PROD-*rol/rol* mice showed a 318-bp fragment (Fig. 2B). Sequence analyses of the PCR products showed that the retroposon-like element was inserted in intron 1 (data not shown). We found the retroposon-like element in both male and female PROD-*s/s* and PROD-*rol/rol* mice (data not shown).

***Ednrb* gene expression levels in the colon:** Real-time qRT-PCR analysis was performed to determine the levels of *Ednrb* mRNA expression in enteric neurons of PROD-*s/s*, PROD-*rol/rol* and B6 mice (Fig. 2C). The relative expression level of *Ednrb* was not significantly different between PROD-*s/s* and PROD-*rol/rol* mice, both of which showed

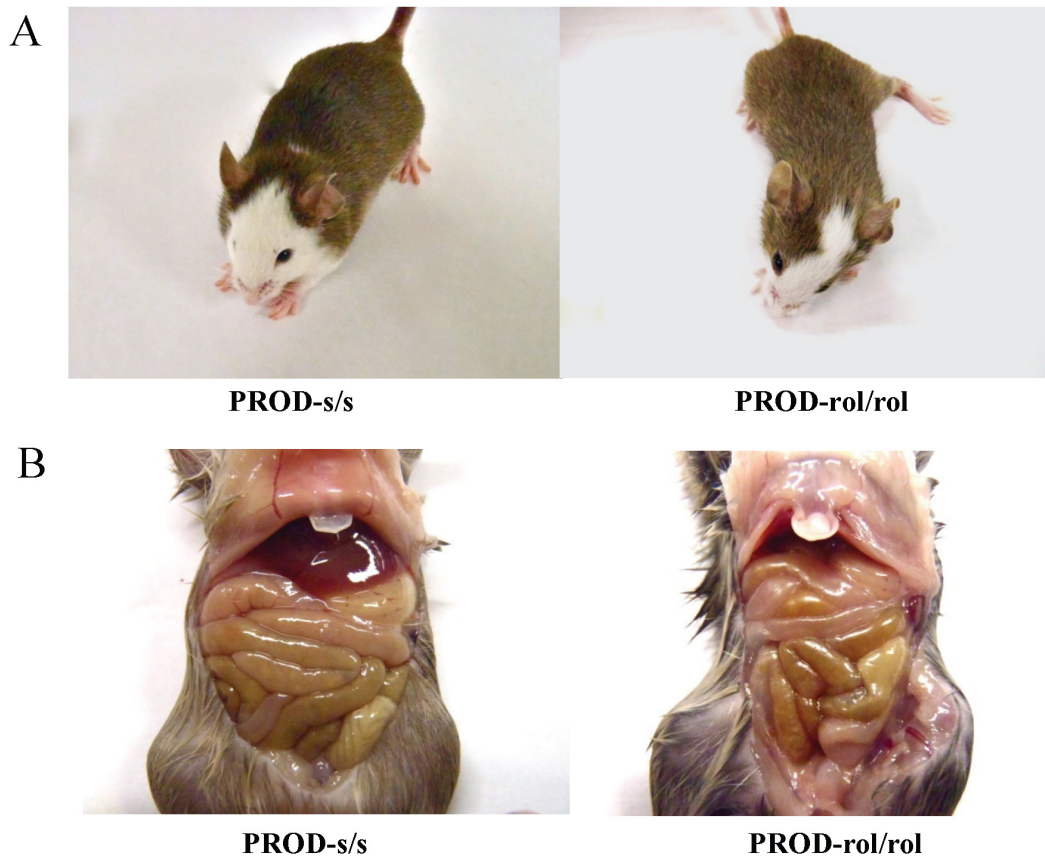


Fig. 1. Phenotypes of PROD-s/s and PROD-rol/rol mice. (A) Representative photographs showing white spots on agouti coat of PROD strain and ataxia of PROD-rol/rol mice are presented. (B) Representative photographs of the open abdomen of PROD-s/s and PROD-rol/rol mice are shown.

about 25% lower levels of *Ednrb* expression in the colon compared to control B6 mice.

***Cacnala* gene expression patterns in the colon:** To examine whether mutation of the *Ednrb* gene affects the *Cacnala* expression pattern in the colon, we used *in situ* hybridization and real-time qRT-PCR analyses. The results of *in situ* hybridization analysis indicated similar patterns of *Cacnala* mRNA expression in the colon of PROD-s/s, PROD-rol/rol and B6 mice (Fig. 3A). Using the antisense probe, all three mouse strains were shown to express *Cacnala* in enteric neurons. No signals were seen in the colon using the sense probe in any of these strains (data not shown). Real-time qRT-PCR analysis was performed to determine the levels of *Cacnala* mRNA expression in enteric neurons in the three mouse strains (Fig. 3B). The relative levels of *Cacnala* mRNA were not significantly different between PROD-s/s and PROD-rol/rol mice, but they were both significantly lower than that in the colon of B6 mice.

Analysis of microsatellite loci adjacent to the Ednrb locus: To examine the origin of the mutant *Ednrb* gene in PROD-s/s and PROD-rol/rol mice, we typed microsatellite loci on chromosome 14. The location order and distances among these loci are presented in Fig. 4A. As shown in Fig. 4B,

fragments were amplified from PROD-s/s, PROD-rol/rol and B6 mice. According to MMDBJ and MGI, the different sizes between JF1 and B6 strains are produced in *D14Mit30* (JF1, B6: size=116 bp, 154 bp), *D14Mit193* (JF1, B6: size=134 bp, 119 bp), *D14Mit93* (JF1, B6: size=189 bp, 147 bp), *D14Mit94* (JF1, B6: size=108 bp, 104 bp), *D14Mit170* (JF1, B6: size=163 bp, 146 bp), *D14Mit42* (JF1, B6: size=142 bp, 152 bp) and *D14Mit267* (JF1, B6: size=97 bp, 114 bp) loci. Amplified fragments of the same length were obtained in both male and female PROD-s/s and PROD-rol/rol mice (data not shown).

DISCUSSION

The results of the present study indicated that the *rolling* Nagoya strain PROD-rol/rol, which exhibits white spots on the agouti coat, has two mutations in the *Ednrb* gene, i.e., two C to T nucleotide substitutions at positions 159 and 1032 in the coding region without amino acid substitutions and a retroposon-like element insertion into intron 1. These mutations have been reported to be piebald spotting (*s*) mutations [4, 6, 9, 20].

In addition to the coat pigment defect, piebald lethal (*s*^l/

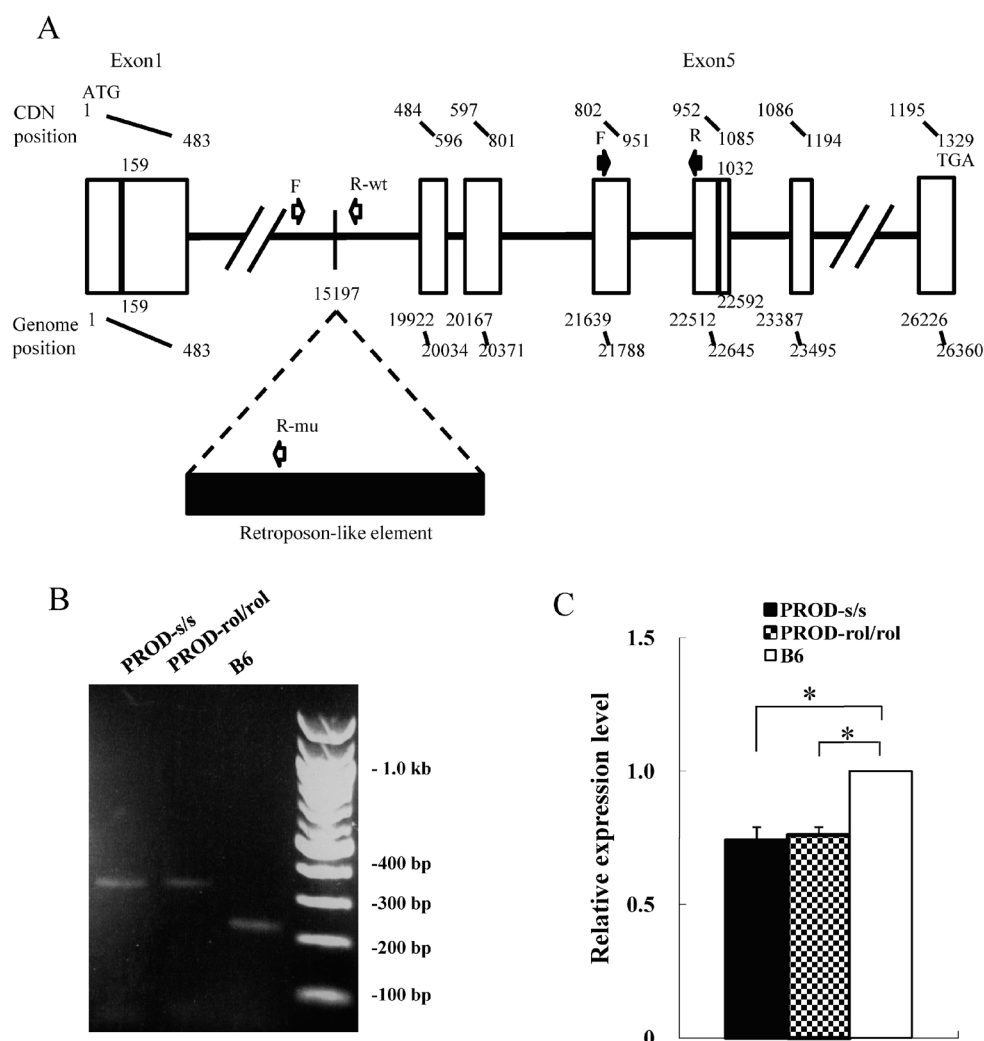


Fig. 2. Structure and expression of the *Ednrb* gene. (A) Exons are shown as white boxes, and the retroposon-like region is shown as a black box. White arrows represent the primers used to amplify the normal (WT) and mutant (Mu) alleles by PCR as previously reported [4]. Black arrows represent the primers used to examine mRNA expression using real-time qRT-PCR. (B) Genotyping results of the WT and Mu alleles are shown. (C) The expression levels of *Ednrb* mRNA in the colon determined by real-time qRT-PCR. The *Ednrb* mRNA expression level for each strain was calculated relative to that in B6 mice. * $P < 0.05$ compared to the appropriate control (Dunnett's test).

s^l mice, which carry a severe mutation at the s locus and lack the full-length transcript of the *Ednrb* gene, show the megacolon phenotype [6]. The s^l/s^l , s/s^l , s/s and $s^l/+$ mice, in which the relative ratios of *Ednrb* expression are about 0%:12.5%:25%:50%, show a "graded" coat color phenotype in the extent of white spotting and have a white coat on >95%, 40–50%, ~20% and 0% of the body surface area, respectively [6]. The megacolon phenotype occurs in s^l/s^l mice, but not in s/s^l , s/s or $s^l/+$ mice [6]. These results indicate that the two neural crest-derived cell lineages require different minimal threshold levels of *Ednrb* gene expression. More than 50% of the wild-type level of *Ednrb* density is likely needed for normal development of epidermal melanocytes, whereas at

least 12.5% of the wild-type level is required for a functional enteric nervous system. In the present study, PROD-rol/rol and PROD-s/s mice showed approximately 25% lower levels of *Ednrb* gene expression compared with B6 mice that have an intact *Ednrb* gene. The white-spotted area on the body surface was detected in PROD- s/s and PROD-rol/rol mice without a megacolon.

Regarding the origin of the laboratory mouse, while most regions of the chromosomes and mitochondrial DNA are of *Mus musculus domesticus* origin, the Y chromosome and some particular regions of the autosomes are of *M. m. musculus* or *M. m. molossinus* origin [13]. The C to T nucleotide substitutions at position 1032 in the coding region of the

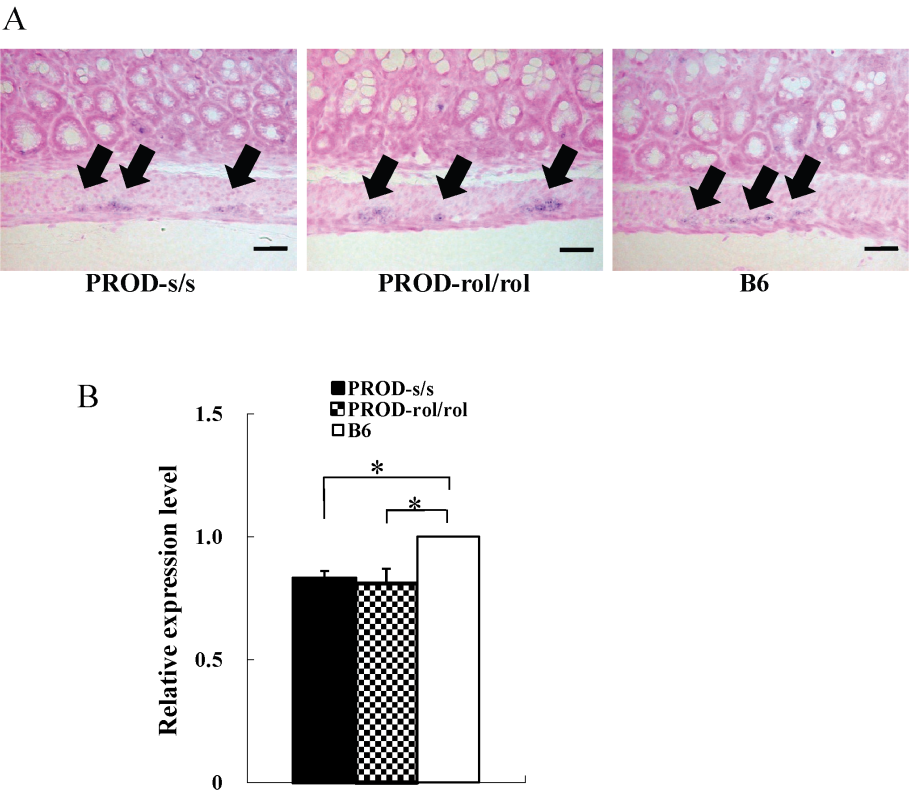


Fig. 3. Expression patterns of *Cacna1a* mRNA in the colon of PROD-s/s, PROD-rol/rol and B6 mice. (A) Representative photographs indicating the localization of *Cacna1a* mRNA in the colon are shown. Arrows indicate enteric nerve plexus. The scale bar is 50 μ m. (B) The expression levels of *Cacna1a* mRNA in the intestine determined using real-time qRT-PCR analysis are shown. The *Cacna1a* mRNA expression level for each strain was calculated relative to that in B6 mice. * P <0.05 compared to the appropriate control (Dunnett's test).

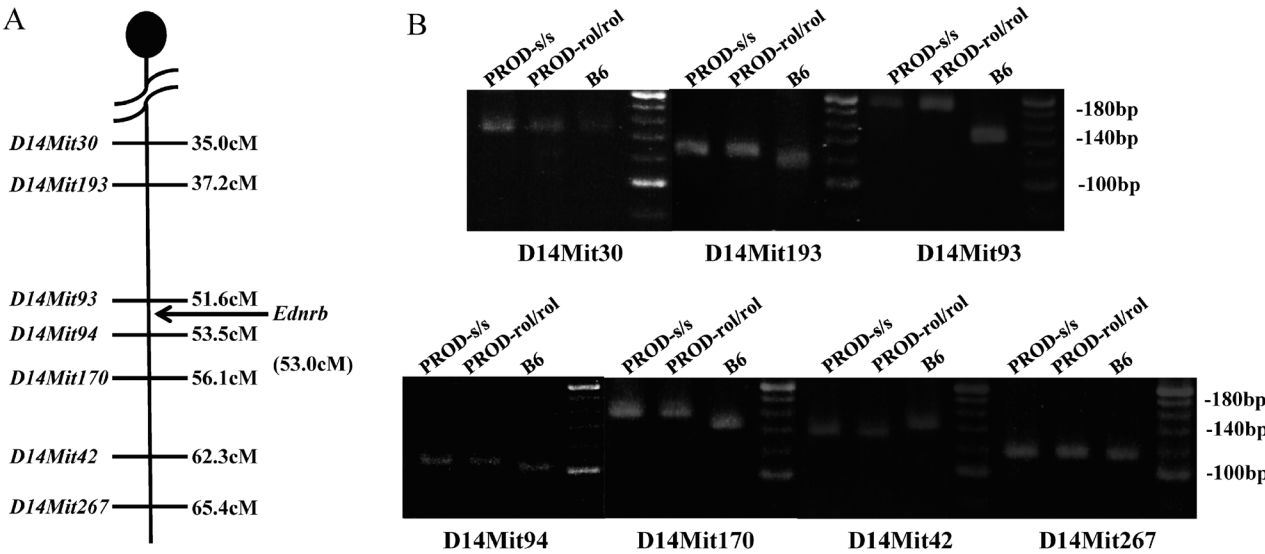


Fig. 4. Microsatellite genotyping. (A) Linkage map of mouse chromosome 14, including the *Ednrb* and microsatellite loci used in this study, is presented. (B) Genotyping results of the microsatellite makers are shown.

Ednrb gene are unique to *M. m. molossinus* origin [9]. JF1 is thought to be derived from the Japanese wild mouse via *M. m. molossinus* [8]. JF1 mice show irregular black spots on the white coat body without a megacolon and have the piebald *s/s* mutation [4]. The *Ednrb* gene is located between the *D14Mit93* and *D14Mit42* loci [9]. The region between *D14Mit93* and *D14Mit42* loci was identical to that of the *M. m. molossinus* strain [9]. These results indicate that the sequence around the *s* locus is derived from *M. m. molossinus*. In the present study, microsatellite genotyping indicated that the alleles of the *D14Mit193*, *D14Mit93*, *D14Mit94*, *D14Mit170* and *D14Mit42* loci were identical to those of the JF1 strain, but that *D14Mit30* and *D14Mitw267* loci were identical to those of the B6 strain. JF1 strain was derived from fancy mice purchased at a market in Denmark in 1987 and established as inbred strain in 1993 [8]. PROD strain was derived from the descendants of (SIII x C57BL/6) F1 and discovered in 1969 [14]. Although the genetic background of the SIII strain has not been reported, our results indicated that at least the region of chromosome 14 between the *D14Mit193* and *D14Mit42* loci of the JF1 and PROD strains was derived from the Japanese fancy piebald mouse via *M. m. molossinus*.

The results of this study indicate that PROD-*rol/rol* is a double-mutant strain with an amino acid change at R1262G in the *Cacna1a* gene on chromosome 8 causing $\text{Ca}_v2.1$ channel dysfunction and the classic piebald mutation in the *Ednrb* gene on chromosome 14 causing the coat pigment defect. Expression analyses indicated that the mutation in the *Ednrb* gene did not affect the expression patterns of $\text{Ca}_v2.1\alpha_1$ in the colon of mice with the PROD genetic background. Previous immunohistochemical experiments indicated that JF1 mice showed a lower density of enteric neurons in the colon compared to B6 mice [4]. In the present study, the level of $\text{Ca}_v2.1\alpha_1$ expression in the PROD strain was lower than that in the B6 strain, suggesting lower enteric neuronal density due to the classic piebald mutation. The $\text{Ca}_v2.1$ currents are acquired during neuronal differentiation from neural crest [3]. Acetylcholine release at the peripheral neuron muscular junctions in the colon depends on calcium channels, including $\text{Ca}_v2.1$ channels [18]. To understand the functions of $\text{Ca}_v2.1$ in the *rolling* Nagoya mouse strain, establishing a strain with an intact *Ednrb* gene will be important.

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